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Species Variation in Osmotic, Cryoprotectant, and Cooling Rate Tolerance in Poultry, Eagle, and Peregrine Falcon Spermatozoa[1]

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ABSTRACT

Potential factors influencing spermatozoa survival to cryopreservation and thawing were analyzed across a range of the following avian species: domestic chicken (*Gallus domesticus*), domestic turkey (*Meleagris gallopavo*), golden eagle (*Aquila chrysaetos*), Bonelli's eagle (*Hieraaetus fasciatus*), imperial eagle (*Aquila adalberti*), and peregrine falcon (*Falco peregrinus*). Studies focused on spermatozoa tolerance to the following: 1) osmotic stress, 2) different extracellular concentrations of the cryoprotectant dimethylacetamide (DMA), 3) equilibration times of 1 versus 4 h, 4) equilibration temperature of 4 versus 21°C, and 5) rapid versus slow cooling before cryopreservation and standard thawing. Sperm viability was assessed with the live/dead stain (SYBR-14/propidium iodine). Sperm viability at osmolalities ≥800 mOsm was higher ($P < 0.05$) in raptor than poultry semen. Return to isotonicity after exposure to hypertonicity (3000 mOsm) decreased ($P < 0.05$) number of viable spermatozoa in chicken, turkey, and golden and Bonelli's eagle spermatozoa but not in imperial eagle or peregrine falcon spermatozoa. Differences were found in spermatozoa resistance to hypotonic conditions, with eagle species demonstrating the most tolerance. Semen, equilibrated for 1 h (4°C) in diluent containing DMA (≥2.06 M), experienced decreased ($P < 0.05$) spermatozoa survival in all species, except the golden eagle and peregrine falcon. Number of surviving spermatozoa diminished progressively with increasing DMA concentrations in all species. Increased equilibration temperature (from 4 to 21°C) markedly reduced ($P < 0.05$) spermatozoa survival in all species except the Bonelli's eagle and turkey. Rapid cooling was detrimental ($P < 0.05$) to spermatozoa from all species except the imperial eagle and the chicken. These results demonstrate that avian spermatozoa differ remarkably in response to osmotic changes, DMA concentrations, equilibration time, temperature, and survival after fast or slow freezing. These differences emphasize the need for species-specific studies in the development and enhancement of assisted breeding for poultry and endangered species.

male sexual function, seasonal reproduction, sperm, sperm motility and transport

INTRODUCTION

Despite extensive research on cryogenic spermatozoa storage in domestic [1–3] and nondomestic [4–9] avian species, there has been limited application to on-farm use or enhanced management for conserving rare, nondomestic species. Dimethylacetamide (DMA) and dimethylsulfoxide (DMSO) have been used as alternative cryoprotectants to glycerol because of its contraceptive effect [10]. However, associated protocols remain troublesome because of the need for many steps in the freeze-thaw process. Additionally, inseminations with thawed spermatozoa produce fewer fertile eggs than do inseminations with fresh semen [2, 5, 11].

Efforts to cryopreserve chicken and turkey spermatozoa have been relatively successful in terms of fertility after artificial insemination (AI). Lake and Stewart [12] reported 53% fertility in the chicken after a single deep intravaginal insemination of 600×10^6 spermatozoa. Considerably higher fertility (~90%) has been obtained when sperm were inseminated at 3-day intervals or on 3 consecutive days [13, 14]. These results were obtained using semen equilibrated and then cooled at 1°C/min to −35°C, followed by liquid nitrogen immersion. Similar success has been reported with chicken and turkey spermatozoa using DMA as cryoprotectant and after pellet freezing [15]. These reports provided little information about the loss of fertilizing ability from cryopreservation. Furthermore, when less massive numbers of spermatozoa are inseminated, it is commonly known that cryopreservation reduces spermatozoa fertilizing ability by >85% (chicken) [16].

Similar problems have been encountered with cryopreserved spermatozoa in the few wild species studied. Artificial insemination in the greater Sandhill crane with spermatozoa cryopreserved in 6% DMSO yields 50% fertile eggs [5], compared with 95% fertility using the same number of fresh spermatozoa. A study of the American kestrel has revealed a 26.1% reduction in fertility using 12.3% DMA as cryoprotectant, compared with using fresh semen [11].

Traditional empirical methods (modifying technical conditions in freezing trials) have been investigated intensely for more than four decades in birds as well as mammals and are unlikely to achieve further significant progress [17]. Alternatively, phenomenological and mechanistic models have been proposed to predict the likelihood of freezing-induced injury or mortality [18, 19]. Whether the sperm membrane ruptures as a result of intracellular nucleation or whether osmotic stress promotes cell destruction is still debated. Nonetheless, one unifying theme has emerged; that is that cryobiology has been successful in beginning to understand the fundamental mechanisms related to how living cells respond to the lowering of temperature [20]. Mem-

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brane permeability to water and permeating cryoprotectants, time of exposure, resistance to osmotic changes, minimal critical volume, and cell size and morphology greatly influence the magnitude of freezing injury. Such basic data have laid the foundation for developing routine and effective cryopreservation protocols of human [21], bull [22], and stallion [23] spermatozoa.

The present study was designed to analyze these same factors in the bird spermatozoa. A comparative approach across a variety of avian species was used largely to determine cross-taxa variation in physical-chemical requirements and in tolerance to cooling/freezing stressors. Two common poultry species (chicken and turkey) were chosen, with comparative analysis being enhanced by having unique access to multiple rare raptor species in which males were trained to provide semen. The predictions were 1) that these spermatozoa characteristics would be at least modestly conserved among species and 2) that specific information on biophysical membrane properties and the response of spermatozoa to certain freezing conditions would eventually provide clues to spermatozoa cryopreservation in a variety of species. This information would help develop genetic resource banks to enhance the management of poultry and endangered bird species.

MATERIAL AND METHODS

Species and Animals Used

Representative species of the Galliform and Falconiform orders were studied. The former included the chicken (*Gallus domesticus,* n = 10) and turkey (*Meleagris gallopavo,* n = 40). The latter included the golden eagle (*Aquila chrysoetos,* n =4), Bonelli's eagle (*Hiernaetus fasciatus,* n = 2), imperial eagle (*Aquila adalberti,* n = 2), and peregrine falcon (*Falco peregrinus,* n = 4). Chickens were maintained in individual cages, whereas turkeys were housed in groups of 10. These two species were exposed to an artificial photoperiod (14L:10D). For nondomestic counterparts, only adult, paired, reproductively active males previously known to consistently produce uncontaminated semen were chosen. All raptors were housed in outdoor pens (6 × 610 m) under a natural photoperiod and located in close proximity of one another, with maximum distance between pens no more than 50 m. Caretakers, feeding schedules and handling of birds was kept constant among all birds. Eagle ages varied from 11 to 23 yr (normal adult age, 4 to 20 yr for the Bonelli's eagle and 5 to 28 in the golden and imperial eagles); falcons ranged in age from 3 to 13 yr (normal adult age, 3 to 16 yr). Raptor species were chosen so that their maximal semen production periods overlapped; the breeding seasons of the imperial eagle, golden eagle, and peregrine falcon are similar. In the south of Spain, the Bonelli's eagle natural breeding season begins earlier than the other species; however, because the raptor facility is located in central Spain, their breeding season is delayed. Therefore, semen from all four of these species could be collected and compared on a given day.

Semen Collection, Evaluation and Sample Preparation

Semen was collected from poultry species by massage as described by Quinn and Burrows [24]. This same technique was adapted for eagles [25] and the peregrine falcon [26]. Semen was collected from individuals once per day for 5 days per week during the spermatozoa production period. All raptors were brought to a lab located in the middle of the facility for semen collection; therefore, semen was evaluated within 5 min of collection from all birds. Samples were discarded if contaminated with urine or fecal material. Semen was collected at field temperature in glass vials or glass capillary tubes. Ejaculates from males of the same species were pooled immediately after collection, and spermatozoa concentration was determined from the pool with a hemocytometer. Chicken spermatozoa was diluted 1:2 with the following diluent: D-fructose (0.8 g), protamine sulphate (0.032 g), sodium glutamate (1.92 g), potassium acetate (0.5 g), polyvinylpyrrolidone (M_r 10 000; 0.3 g), and 100 ml H_2O; pH 6.85, 313 mOsm. Fresh turkey semen was diluted 1:3 with the diluent: D-fructose (1.15 g), sodium glutamate (2.1 g), polyvinylpyrrolidone (M_r, 10 000; 0.3 g), glycine (0.2 g), potassium acetate (0.5 g), and 100 ml H_2O; pH 6.65, 371 mOsm. Semen pool from every raptor species was diluted at room temperature with Lake diluent (100 mM sodium glutamate, 44 mM fructose, 4 mM magnesium acetate, 51 mM potasium acetate, and 30 μM polyvinylpyrrolidone; 300 mOsm [27], and concentration was adjusted to 0.5×10^6 spermatozoa/ml for all species. Semen was maintained at room temperature in open Eppendorf vials. All dilutions were made at room temperature.

Sperm Viability Assessment

Sperm viability was assessed in both fresh (undiluted) and altered (diluted) sperm suspensions by using the live/dead stain combination (SYBR-14/propidium iodine [PI], Fertilight Kit, Molecular Probes, Eugene, OR) with minor adaptations by Donoghue et al. [28]. Seminal aliquots (5 μl) were added to 50-μl solutions containing 5 μl PI (diluted 1:100 in the species diluent) and 1 μl SYBR-14 (diluted 1:100 in DMSO). When semen maintained under hyper- or hypotonic conditions required evaluation, SYBR-14-PI solutions were made at the same final osmolarity required for each study. Assessment of 100 spermatozoa were made in duplicate aliquots for every sample and evaluated microscopically at 40× by using an Axioskop microscope and fluorescein isothiocyanate (FITC) filter fluorescence. SYBR-14, a membrane-permeant DNA stain, stained only living spermatozoa, producing bright green fluorescence of the nuclei when excited at 488 nm. Propidium iodide stained the nuclei of membrane-damaged cells red.

Study Designs and Sample Treatment

All experimental trials were run three times for each species and DMA concentration. Spermatozoa from each species were subjected to seven different studies to evaluate sperm membrane properties and to compare membrane damage (i.e., viability).

Study 1: Resistance to hyperosmotic stress. NaCl was added to the isosmotic species-specific diluents (described above) to achieve 500, 816, 1500, or 3000 mOsm solutions. The final osmolality of each was checked with a vapor pressure osmometer (5500 Wescor, Inc., Logan, UT). Sperm suspensions and the hyperosmotic solutions were equilibrated to 21°C, and 2-μl sperm aliquots from every species were added and mixed rapidly with 50 μl of each hyperosmotic solution. After 10 min, three 5-μl aliquots from each solution were evaluated for sperm viability.

Study 2. Susceptibility to hypotonic stress. Three aliquots (5 μl each) of sperm solution from each species were added to Eppendorf vials containing 50 μl of a 50-mOsm

TABLE 1. Specific differences in fresh sperm viability (%) after a 10-min exposure to hyper- or hypotonic conditions, compared with isotonic conditions (300 mOsm).[a]

Osmotic conditions (mOsm)	Chicken (n = 10)	Turkey (n = 40)	Golden eagle (n = 4)	Bonelli's eagle (n = 2)	Imperial eagle (n = 2)	Peregrine falcon (n = 4)
50	22.5 ± 3.4[d]	13.8 ± 2.7[b]	76.3 ± 1.9[d]	55.1 ± 3.1[c]	41.8 ± 3.4[e]	2.8 ± 1.4[e]
300	100[b]	100[c]	100[b]	100[b]	100[b]	100[b]
500	96.8 ± 2.3[b]	92.3 ± 2.8[c]	97.6 ± 2.0[b]	100[b]	95.1 ± 1.7[b]	100[b]
800	73.6 ± 2.9[c]	81.8 ± 2.7[d]	92.3 ± 3.2[b,c]	97.6 ± 2.0[b]	96.0 ± 1.4[b]	97.1 ± 2.4[b]
1500	71.8 ± 2.9[c]	81.0 ± 4.09[d]	95.3 ± 1.5[b]	95.5 ± 2.0[b]	93.1 ± 2.4[c]	95.5 ± 2.0[c]
3000	70.3 ± 3.3[c]	61.1 ± 3.1[e]	91.0 ± 2.0[c]	96.8 ± 2.3[b]	89.3 ± 3.0[d]	90.8 ± 2.6[d]

[a] Data are normalized to the percentage of viable sperm at 300 mOsm and represent means ± SD.
[b–e] Different superscripts represent differences ($P < 0.05$) within columns, within species.

NaCl solution. Semen was evaluated for viability after 10 min.

Study 3: Spermatozoa sensitivity after restoration of isotonicity after hypertonic exposure. To test the impact of rapid return to isotonicity, three aliquots (5 μl each) from the 3000-mOsm sperm solutions (produced in study 1) were immediately added in a single step to chicken, turkey, or raptor diluents (50 μl as above, with osmolality adjusted to 300 mOsm) as appropriate for each species/taxon. Sperm plasma membrane integrity was assessed after 10 min. This study was performed at 21°C to simulate the thawing and rewarming process before artificial insemination.

Study 4: Spermatozoa membrane permeability to DMA as by equilibration time before freezing. Sperm solutions from each species were diluted 1:1 in the respective species/taxon diluent to give the following final DMA concentrations: 0.68, 1.37, 2.06, 2.70, and 3.44 M. Spermatozoa were equilibrated for 1 and 4 h (at 4°C), after which three 5-μl samples per individual were evaluated for viability.

Study 5: Equilibration temperature as a factor modifying membrane permeability to DMA. Dimethylacetamide solutions (0.68 and 3.44 M) were added to diluted semen from all species (except the chicken). Aliquots from the same sample were equilibrated for 1 h at 4 or 21°C, and then spermatozoa were evaluated for viability.

Study 6: Spermatozoa survival after rapid versus slow cooling. Spermatozoa from each species were diluted as described in study 4 and equilibrated for 1 h (at 4°C) in plastic cryovials. Each sample was independently cryopreserved using a rapid or slow cooling rate. Rapid cooling consisted of plunging cryovials into liquid nitrogen (50°C/min). For slow cooling, rates of 1°C/min (from +4°C to −20°C), followed by 2°C/min (from −20°C to −70°C), followed by liquid nitrogen immersion were used. After 24 h of liquid nitrogen storage, all samples were thawed at 4°C, and then 5-μl aliquots from each sample were evaluated for sperm viability.

Statistical Analysis

Within species, one factor and multifactor analysis of variance (ANOVA) using the least squares procedure and the general linear models procedure of the Statistical Analysis System [29] were performed, and the a priori-determined significance level was $P < 0.05$. Values were arcsine transformed for analysis. Percentage data were arcsine transformed before analysis, and significance was based on transformed means. Data are displayed as arithmetic means ± SD for clarity of presentation.

RESULTS

Spermatozoa Tolerance to Anisosmotic Conditions (Studies 1 and 2)

Regardless of species, moderately increasing osmolality of the dilution medium (to 500 mOsm from an isotonicity of 300 mOsm) had a negligible ($P > 0.05$) effect on sperm viability (Table 1). However, at osmolalities ≥800 mOsm, chicken and turkey spermatozoa were adversely affected ($P < 0.05$), but raptor spermatozoa were not. The eagle species and peregrine falcon spermatozoa tolerated extreme hyperosmotic conditions (10× greater than isotonicity) as viability was maintained from 89.3 ± 3% (imperial eagle) to 96.8 ± 2.3% (Bonelli's eagle, Table 1). The sperm viability declines were small but significant ($P < 0.05$) at 1500 and 3000 mOsm for the imperial eagle and peregrine falcon. The decline at 3000 mOsm for the golden eagle was also significant ($P < 0.05$). With the exception of the chicken and the Bonelli's eagle, NaCl induced hyperosmolality caused posthypertonic spermolysis, with the turkey being the most sensitive species.

Spermatozoa from eagle species also demonstrated the greatest tolerance to hypotonic (50 mOsm) conditions, with sperm viability ranging from 41.8 ± 3.4% (imperial eagle) to 76.3 ± 1.0% (golden eagle, Table 1). In contrast, viability was markedly reduced ($P < 0.05$) in chicken and turkey spermatozoa with the highest mortality (97.2%), occurring in peregrine falcon spermatozoa.

Spermolysis Induced by Return to Isotonicity (Study 3)

After exposure to hypertonic conditions and return to isotonicity, sperm viability decreased ($P < 0.05$) in all species but the imperial eagle and peregrine falcon. Turkey spermatozoa appeared least tolerant to return to isotonicity, with an average 13% decline in survival (Fig. 1).

Specific DMA Permeability and Temperature Influence (Study 4)

Table 2 depicts the portion of viable spermatozoa in each species after 1 and 4 h (at 4°C) in diluent containing varying DMA concentrations. Concentrations through 1.37 M DMA had no effect ($P > 0.05$) on spermatozoa from any species when the equilibration interval was 1 h. However, DMA at 2.06 M decreased spermatozoa survival ($P < 0.05$) in the chicken, turkey and imperial eagle. Sperm viability in all species decreased ($P < 0.05$) at DMA concentrations above 2.06 M in a progressive and similar fashion.

In almost all cases, prolonging the equilibration to 4 h accelerated the rate of cell death and lowered the proportion of viable spermatozoa (Table 2). The impact was most pro-

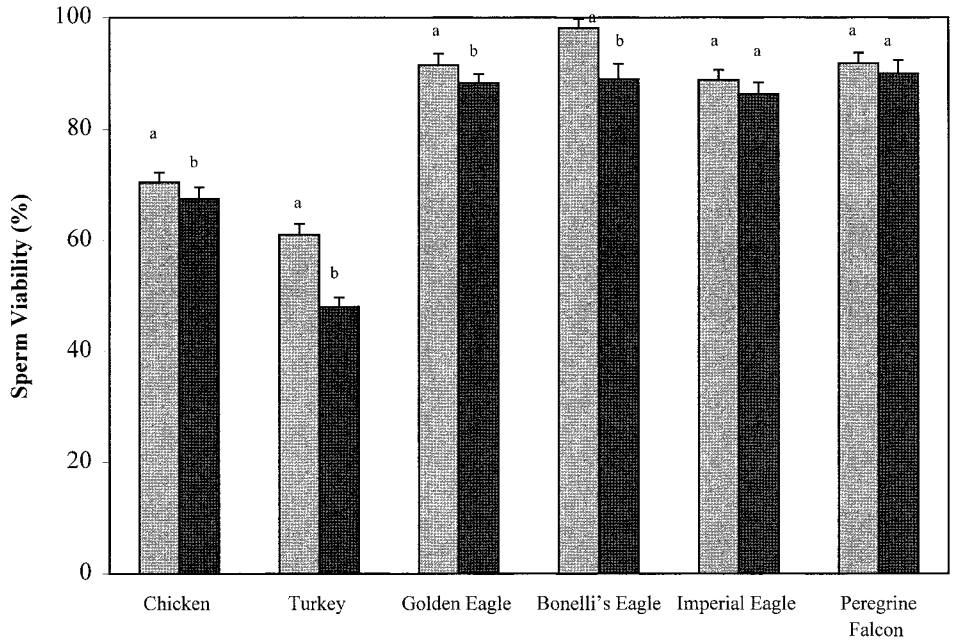

FIG. 1. Comparative effect of restoration to (300 mOsm, light bars) isotonicity on sperm viability (%) after hypertonic exposure (3000 mOsm, dark bars) in poultry and raptors. Data are normalized to the percentage of viable sperm at 300 mOsm before treatment. Values are means ± SD; superscripts indicate difference ($P < 0.05$) between paired columns.

found in the peregrine falcon, for which no spermatozoa survived for 4 h in ≥2.06 M DMA. In contrast, sperm viability patterns in imperial eagle spermatozoa at 4 h were similar to those at 1 h, regardless of DMA concentration.

Impact of Equilibration Temperature (Study 5)

For the lower (0.68 M DMA) treatment, refrigeration temperature (4°C) was detrimental to sperm viability when compared with room temperature treatment (21°C) for three (Bonelli's and imperial eagles and peregrine falcon) out of the five species examined ($P < 0.05$; Fig. 2). With the exception of the turkey and peregrine falcon, with higher (3.44 M) DMA concentration, increasing equilibration temperature from 4 to 21°C markedly reduced ($P < 0.05$) sperm viability (Fig. 2). Interactions between temperature and DMA concentration were nonsignificant ($P > 0.05$) for all species.

Effect of the Cooling Rate on Spermatozoa Survival (Study 6)

Rapid cooling was detrimental to spermatozoa from all species except the imperial eagle and the chicken (Fig. 3). For the chicken and imperial eagle, percentage of viable spermatozoa after thawing increased ($P < 0.05$) when rapid, compared with slow, cooling was used (Fig. 3, a and c). With rapid cooling, spermatozoa survival increased progressively for imperial eagle as DMA concentration increased (Fig. 3c). In contrast, slow cooling Bonelli's eagle spermatozoa as DMA concentration increased reduced viability (Fig. 3d). The opposite occurred in turkeys; slow cooling in increasing DMA increased sperm viability (Fig. 3b).

DISCUSSION

Artificial insemination with cryopreserved spermatozoa has long been recognized as important to poultry production for food [30]. This assisted-breeding approach has enormous potential for managing endangered populations, especially 1) in maintaining heterozygosity in small populations, 2) in combating sexual incompatibility, and 3) avoiding depletion of wild stocks by relying on germ plasm rather than removing animals from nature [31, 32]. For more than two decades, AI (and other assisted-breeding strategies) have been touted as potentially useful for managing endangered species. However, it has become apparent that this general assumption is overstated [33], in part because of species variations in reproductive mechanisms, like the profound differences in cellular reproductive traits measured in the present studies. In this case, osmotic, cryoprotectant, and cooling-rate tolerance varied markedly among avian species, even those closely related taxonomically. Although the basic data are of fundamental interest, perhaps most important is the implication of our findings to the practical application of AI with frozen spermatozoa. It is obvious that there will be a need for significant species-specific research before such assisted-breeding techniques can be used routinely to help manage wild bird species.

Conventional avian sperm cryopreservation involves considering all aspects of anisosmotic events that essentially regulate membrane integrity during freezing-thawing [21]. Cryoprotectant and cooling rate also influence final cell viability after thawing. The present studies revealed that 1) raptor spermatozoa tolerated hyperosmotic conditions better than that of poultry species; 2) restoration of isotonicity adversely affected sperm viability in certain species; 3) prolonged equilibration at refrigeration temperatures benefited sperm viability for some, but not all, species; and 4) an optimal cooling rate may exist for every species independent of other factors evaluated here.

To date, sperm cryopreservation studies in birds have focused on poultry with the occasional application of knowledge directly to wild species. Most results in wild species reported fertility success after AI [5, 8, 11]. Our approach here was different and was based on the speculation that 1) specific differences existed in sperm mem-

TABLE 2. Sperm viability in samples equilibrated at 4°C for 1 h or 4 h in diluents containing different DMA concentrations.[a]

	Chicken (n = 10)		Turkey (n = 40)		Golden eagle (n = 4)	
DMA (M)	1 h	4 h	1 h	4 h	1 h	4 h
Control	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]
0.68	100[b]	80.2 ± 3[c]	100[b]	100[b]	100[b]	100[b]
1.37	100[b]	86.8 ± 5[c]	100[b]	100[b]	100[b]	89.7 ± 6[c]
2.06	83.3 ± 6[c]	79.3 ± 3[c]	86.6 ± 6[c]	78.0 ± 5[c]	100[b]	88.0 ± 2[c]
2.70	75.0 ± 5[d]	72.5 ± 6[d]	84.4 ± 4[c]	70.5 ± 4[c]	84.2 ± 4[c]	74.3 ± 3[d]
3.44	71.0 ± 7[d]	57.0 ± 6[e]	65.9 ± 3[d]	61.0 ± 4[d]	81.4 ± 5[c]	51.2 ± 3[e]
	Bonelli's eagle (n = 2)		Imperial eagle (n = 2)		Peregrine falcon (n = 4)	
DMA (M)	1 h	4 h	1 h	4 h	1 h	4 h
Control	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]
0.68	100[b]	100[b]	100[b]	100[b]	100[b]	50.0 ± 3[c]
1.37	100[b]	100[b]	100[b]	100[b]	100[b]	56.2 ± 6[c]
2.06	93.2 ± 5[b]	80.0 ± 6[c]	75.0 ± 5[c]	84.3 ± 3[c]	100[b]	0[d]
2.70	84.7 ± 5[c]	80.0 ± 6[c]	73.1 ± 7[c]	80.1 ± 4[c]	82.3 ± 4[c]	0[d]
3.44	50.8 ± 4[d]	53.3 ± 5[d]	72.3 ± 10[c]	73.0 ± 4[d]	70.5 ± 5[d]	0[d]

[a] Data are normalized to the percentage of viable sperm in diluents without cryoprotectant (control) and represent means ± SD.
[b–e] Different superscripts represent differences ($P < 0.05$) within columns within species.

brane characteristics that could influence tolerance to certain freezing variables and 2) some factors affecting survival to a cryopreservation stress could be isolated and used to identify precise spermatozoa tolerance to each. Such information is fundamental to optimizing protocols to eventually develop species-specific sperm cryopreservation methods resulting in consistently successful assisted breeding.

When the spermatozoa of various species were exposed to identical osmotic conditions (up to 800 mOsm), chicken and turkey cells reacted differently from those of raptors. Apparently, dehydration imposed by extracellular hyperosmotic solutions induced marked, irreversible damage to chicken and turkey sperm membranes. Moreover, our results supported the assertion that remarkable variations must exist in bird sperm membrane permeability to account for the vastly different survival responses to varying osmotic conditions. Raptor species especially were tolerant of extracellular hyperosmolarity. Osmotic tolerance in mammalian spermatozoa is influenced by exposure, temperature, type of solute, and solute concentration [34, 35]. Although no attempt was made to measure sperm volume after treatment, there appeared to be a relationship between posthypertonic spermolysis and the degree of cell water loss, as reported in mammalian spermatozoa. Regardless, the reason for raptor spermatozoa resisting extreme hyperosmotic conditions remains unknown. Restoration to isotonicity caused a slight but significant increase in spermolysis, but again, it was of a magnitude considerably less than in mammal spermatozoa exposed to a similar stress [35]. The exceptions were imperial eagle and peregrine falcon spermatozoa, which apparently had an inherent resistance for maintaining membrane integrity after dehydration followed by rehydration.

It also was noted that sperm viability on the basis of osmotic responses was not completely delineated on the

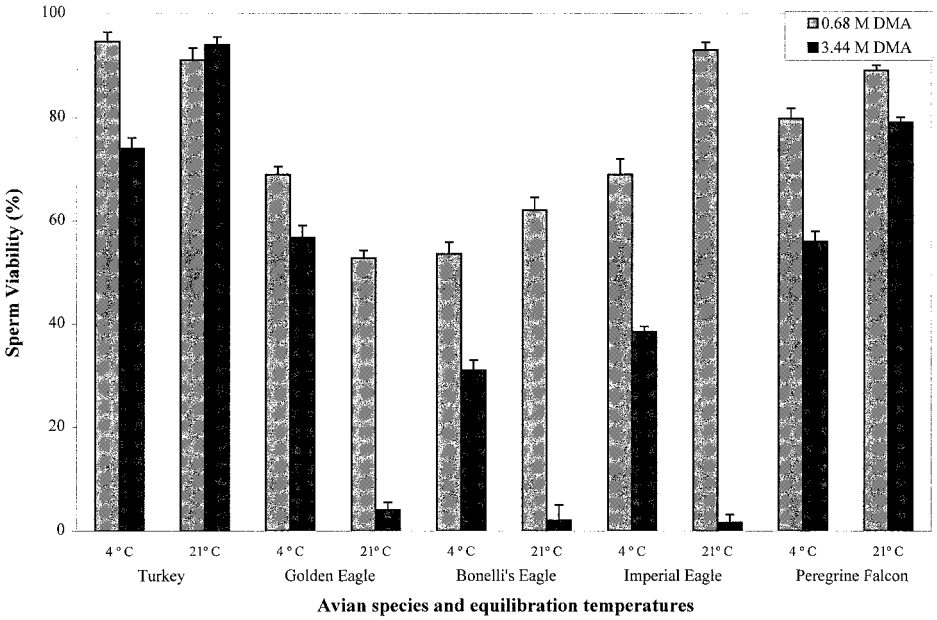

FIG. 2. Comparative effect of increasing equilibration temperatures (4 to 21°C) on sperm viability (%) after 1 h equilibration in medium containing one of two DMA concentrations (0.68 or 3.44 M) in poultry and raptor species. All values are means ± SD.

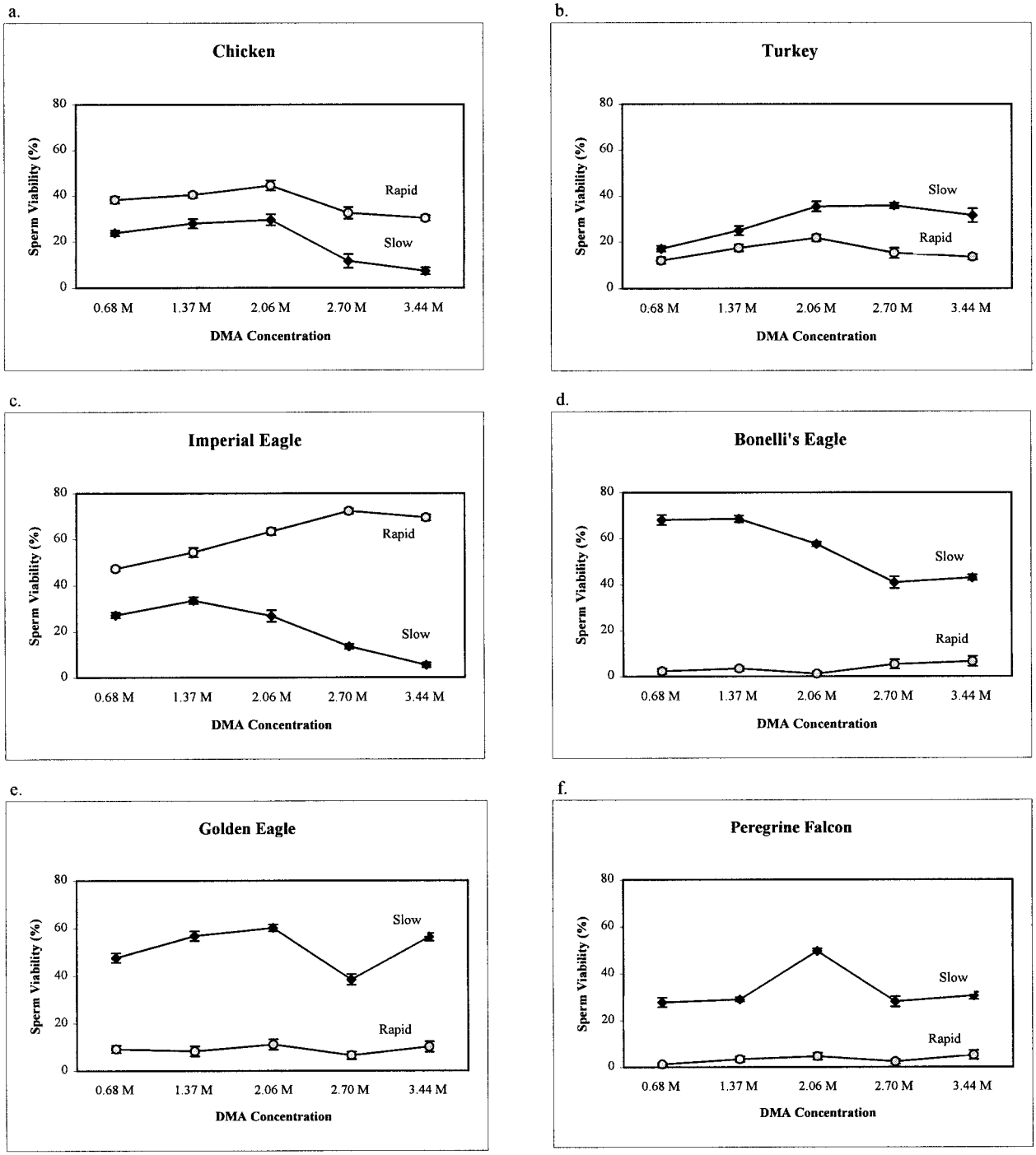

FIG. 3. Sperm viability (%) induced by rapid versus slow cooling of spermatozoa from **a**) chicken, **b**) turkey, **c**) imperial eagle, **d**) Bonelli's eagle, **e**) golden eagle, and **f**) peregrine falcon. The rapid method consisted of plunging cryovials into liquid nitrogen (50°C/min); for the slow method, a rate of 1°C/min (from +4 to −20°C), followed by 2°C/min (from −20 to −70°C), followed by liquid nitrogen immersion was used. After 24 h of liquid nitrogen storage, all samples were thawed at 4°C, and then 5-μl aliquots from each sample were evaluated for sperm viability. Data represent means ± SD.

basis of poultry versus raptors. Significant differences were apparent in the chicken versus turkey, with spermatozoa from the former being more vulnerable to an 800-mOsm experience that then was followed by a rapid loss in survival rate, likely because of an accelerated loss of intracellular water. In contrast, we speculate that the turkey sperm membrane may be less permeable to water, explaining the longer time or higher osmolality needed to reduce sperm viability.

Some of the greatest species variance observed was in sperm response to hypotonic conditions. All eagles (particularly the golden eagle) were especially tolerant compared with the case of poultry. Interestingly, peregrine falcon spermatozoa were not adversely affected by hyperosmolarity, but these cells were highly vulnerable to hypotonicity. Sperm morphology may play a role. Spermatozoa of the peregrine falcon are longer in diameter compared with that of the other species studied here.

Because DMA is a permeating cryoprotectant, spermatozoa exposed to hyperosmotic DMA solutions first shrink

because of dehydration and then swell as DMA and water permeate the cell [36]. DMA concentrations ≥2.70 universally decreased sperm viability in all the species (after 1 h of equilibration at 4°C), with further progressive declines with higher DMA concentrations. Final osmolalities of the 2.70 and 3.44 M solutions were 540 and 570 mOsm, respectively. When comparing turkey sperm viability in DMA or NaCl solutions (with similar final osmolalities and temperature), sperm viability decreased in a similar fashion. However, hypertonicity is a more potent stressor of turkey spermatozoa than excessive DMA concentration. In human spermatozoa, Gao et al. [35] explained this on the basis of membrane permeability. For example, the high permeability of the human spermatozoan to glycerol reduces exposure time to hypertonicity; cell equilibrium is achieved quickly, thereby reducing the opportunity for lysis.

Data revealed an interaction between DMA concentration and interval of incubation in all species, but the imperial eagle. In the other five species, extended exposure to DMA reduced the DMA concentration that optimized viability. This observation strongly suggested that both DMA and NaCl solutions are equally harmful at osmolalities slightly over isotonicity (540 to 570 mOsm) and that the presence of DMA at refrigeration temperature slows the osmotic effects on spermatozoa from certain species.

Again, species specificity strongly influenced the DMA concentration-equilibrium interval relationship, ranging from no effect in the imperial eagle to a severe loss in sperm viability after 4 h incubation of peregrine falcon spermatozoa. The lethality of DMA was not completely associated with spermatozoa susceptibility to hypertonic medium, as demonstrated by lower viabilities at high DMA concentrations (Table 2) compared with that measured in hypertonic NaCl solutions (Table 1). This indicates that DMA toxicity exists and is a phenomenon influenced by equilibration interval. As revealed in study 5, temperature also played a species-specific role in mediating the impact of DMA on spermatozoa survival. In most cases, increasing temperature reduced sperm viability with the odd and opposite (positive) reaction occurring in the peregrine falcon.

In all cell types studied to date, the hydraulic conductivity of the plasma membrane, defined as Lp, decreases with declining temperature [21]. Thus, time to spermolysis increases, which may explain the higher proportions of viable spermatozoa in samples equilibrated at refrigeration temperatures (e.g., golden eagle). Again, spermatozoa from the latter species maintained identical viability from 1–4 h at 4°C in vitro. However, the time to hypotonic spermolysis has been reported to remain essentially constant at temperatures above 0°C in human spermatozoa [21]. In addition, in species like the imperial eagle, the final percentages of viable spermatozoa are extremely reduced at room temperatures. On the basis of the facts mentioned above, the decrease in Lp does not explain the magnitude of the reduction, even when a decrease in the critical water volume is likely to occur with the decrease in temperature.

Assuming the effect of increase in temperature for all the different species is exactly the same, differences in survival may be exclusively due to water flux and the width of the hydrophobic region of the membrane. In species like the peregrine falcon or the turkey, either extraordinary low water permeability or the presence of very thin hydrophobic regions in their membranes might explain the fact that sperm viability at room temperature is significantly superior to other species. Interestingly, in both species, spermatozoa survival significantly increased with increasing temperature. It is possible that for these species, the activation energy for DMA permeability to the falcon sperm membrane is several times higher than that of the water, as proposed in mammals [35].

The ionic environment becomes concentrated during freezing because of the removal of water from the solution in the form of ice, thus creating a driving force for water efflux from the cell [37]. The rate of this efflux depends on the membrane permeability to water. Under certain conditions, like rapid cooling, the cell may not be able to lose enough intracellular water; the cytoplasm becomes supercooled and can freeze, which usually results in a lethal injury [38]. The spermatozoa of certain species, like the imperial eagle or the chicken, may accomplish rapid cooling by maintaining reasonable percentages of viability after thawing; spermatozoa from other species will not survive the process.

Further research is warranted to better understand the biophysical base for the striking differences measured among species in the present study. However, it is now clear that it is imperative to carefully analyze the cryobiological properties of spermatozoa from individual avian species before attempting practical use of assisted breeding.

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